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INCREASED RESPONSIVENESS OF THE HEPATIC GUANYLATE CYCLASE-GUANOSINE 3′,5′-MONOPHOSPHATE SYSTEM TO NITROSOGUANIDINE FOLLOWING PARTIAL HEPATECTOMY

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Summary

When tested at concentrations producing submaximal responses, the *N*-nitroso carcinogen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (methylnitro-nitrosoguanidine) elicited a 2-fold greater increase in guanosine 3′,5′-monophosphate (cyclic GMP) accumulation in slices and a 5-fold greater stimulation of guanylate cyclase activity in whole homogenates of rat liver examined 24 h after 75% hepatectomy compared to the corresponding methylnitro-nitrosoguanidine responses in sham-operated and unoperated controls. Enhanced methylnitro-nitrosoguanidine sensitivity of guanylate cyclase in whole homogenates of regenerating liver was attributable to altered responsiveness of the enzyme activity of the 100 000 × *g* soluble fraction, which contained 98% of the methylnitro-nitrosoguanidine responsive activity. Basal cyclic GMP accumulation and guanylate cyclase activities of these systems, and their responses to concentrations of methylnitro-nitrosoguanidine eliciting maximal stimulation were unchanged after partial hepatectomy or sham operation, compared to unoperated controls. The findings of (a) increased heme concentrations in the supernatant and the high molecular weight Sephadex G-25 fraction of sham operated, compared to regenerating liver, (b) suppression of methylnitro-nitrosoguanidine responsive activity after addition of exogenous hemoglobin to supernatants from regenerating liver, and (c) enhancement of the responsiveness of soluble guanylate cyclase from sham operated liver to submaximal methylnitro-nitrosoguanidine after reduction of endogenous heme content by in situ perfusion, all suggested that the difference in methylnitro-nitrosoguanidine action observed in control vs. regenerating liver are related to a lower

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Abbreviation: MIX, 3-isobutyl-1-methylxanthine; *df*, degrees of freedom.

heme-protein content of the latter. These results emphasize the importance of endogenous heme as a factor modulating the response of the hepatic guanylate cyclase system to methylnitro-nitrosoguanidine.

Introduction

In contrast to several other tissues of the rat, the liver is resistant to the oncogenic effects of a single dose of *N*-nitroso carcinogen [1,2]. Partial hepatectomy enhances the sensitivity of liver to the oncogenic action of *N*-nitroso compounds, as reflected by the fact that carcinomas are induced in liver when a single dose of these agents is administered shortly after partial hepatic resection [3—5]. While alkylation of replicating nucleic acids is thought to play an important role in the enhanced sensitivity of regenerating liver to tumor induction by chemical agents [6,7], the factors responsible for this phenomenon are at present incompletely defined [2,8—10]. In the present study we have examined the effects of partial hepatectomy on responsiveness of rat hepatic guanylate cyclase-guanosine 3′,5′-monophosphate (cyclic GMP) to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (methylnitro-nitrosoguanidine), a known carcinogenic activator of the guanylate cyclase system. The rationale for this study is based on the observations that (1) methylnitro-nitrosoguanidine and other *N*-nitroso carcinogens activate the guanylate cyclase-cyclic GMP systems of numerous mammalian tissues, including liver [11—14]; (2) cyclic GMP has been implicated as a possible determinant of cell growth and transformation [15—22]; (3) partial hepatectomy is known to produce significant alterations both in the properties of the guanylate cyclase system of liver [23—25] and in its sensitivity to *N*-nitroso carcinogens [3—5]; (4) liver heme content is altered during hepatic regeneration [26,27]; and (5) heme has been implicated as an important factor in the expression of the actions of *N*-nitroso compounds and related agents on hepatic guanylate cyclase [28—31].

The results indicate that the sensitivity of the guanylate cyclase-cyclic GMP system of regenerating liver to submaximal stimulatory concentrations of methylnitro-nitrosoguanidine is significantly enhanced compared to that of liver from sham-operated or unoperated controls, probably as a consequence of the diminished heme content of regenerating liver.

Materials and Methods

Chemicals and radiochemicals. Methylnitro-nitrosoguanidine was obtained from Aldrich Chemical Co., Milwaukee, WI. Radiochemicals were obtained from New England Nuclear, Boston, MA. ACS scintillation solution from Amersham Corp., Arlington Heights, IL was used for all scintillation counting. Sources of all other agents have been reported previously [11,12].

Partial hepatectomy. Male Sprague-Dawley rats weighing 200—300 g were obtained from Laboratory Supply, Indianapolis, IN. The partial hepatectomy (70—80%), performed with the rat under ether anesthesia, consisted of resection of the median, left lateral and left caudal lobes [32]. Sham operations consisted of a midline incision with removal of the lower sternum as in hepatec-

tomy, and then closure without further manipulation. Rats were fasted from the time of operation to the time of killing (routinely 24 h). To assess the effect of time after hepatectomy on changes in guanylate cyclase, surgery was performed at the desired intervals prior to the time of sacrifice, with all animals then killed during the same hour. Rats were injected intraperitoneally with 10—25 μCi of [*methyl*-^{3}H]thymidine 1 h prior to removal of livers. DNA was extracted and determined as described previously [33]. At the time of final liver excision, animals were anesthetized with pentobarbital (4 mg/100 g body weight).

Tissue preparation, incubation and assay for cyclic GMP and guanylate cyclase. The resected liver was immediately placed in 0.9% NaCl at 4°C and slices were prepared in the cold room using a Stadie-Riggs microtome. Slices were incubated under conditions described previously [11], and reactions stopped by transferring slices to 0.4 ml of 50 mM sodium acetate at 95°C and heating for 10 min. Following an homogenization, samples were reheated at 95°C for 5 min and centrifuged at 3300 × *g* for 20 min at 4°C. Supernatant cyclic GMP content was determined directly by the radioimmunoassay method of Steiner et al. [34], as reported previously [21,35]. Homogenates, soluble and particulate fractions were prepared from liver slices as described previously [11].

Guanylate cyclase was determined from the conversion of [α-^{32}P]GTP to cyclic GMP, as reported elsewhere [36]. Isolation of cyclic GMP from reaction mixture was accomplished by sequential chromatography on Dowex-50 and alumina [37], using the recent modification of White and Karr [38].

Other determinations. Protein was determined by the method of Lowry et al. [39] using crystalline bovine serum albumin as a standard. Heme concentrations of supernatant fractions were assessed from the Soret band at 415 nm using the formula $(A_{415} - (0.4 \times A_{370}) - (0.6 \times A_{445}))/103$. Absorbance readings at 370 nm and 445 nm allowed for correction of the contribution of light scattering. The method was verified by adding hemoglobin standards to the supernatant fractions.

Significance of differences between means was determined by use of Student's *t*-test for unpaired values.

Results

Methylnitro-nitrosoguanidine responsive cyclic GMP and guanylate cyclase during liver regeneration

As shown in Table I, basal accumulation of cyclic GMP in slices of liver obtained 24 h after partial hepatectomy did not differ from that in sham-operated controls. However, the cyclic GMP response to a submaximal concentration of methylnitro-nitrosoguanidine (100 μM) was significantly enhanced (2-fold over control) following hepatectomy. This difference was evident expressing cyclic GMP accumulation on the basis of tissue wet weight, protein content (Table I) or DNA (not shown). By contrast, responses to a maximally effective concentration of methylnitro-nitrosoguanidine (3 mM) were similar in the two experimental groups. Basal, submaximal and maximal methylnitro-nitrosoguanidine-responsive cyclic GMP following sham operation did not differ from corre-

TABLE I

EFFECT OF PARTIAL HEPATECTOMY ON METHYLNITRO-NITROSOGUANIDINE RESPONSIVE CYCLIC GMP ACCUMULATION IN HEPATIC SLICES

Liver slices were preincubated for 20 min at 37°C in 2 ml of complete Krebs-Ringer bicarbonate buffer solution containing 5 mM glucose and 2 mM MIX, with 95% O_2/5% CO_2 serving as the gas phase. Methylnitro-nitrosoguanidine in 40% acetone was then added to the experimental flasks to give the final indicated concentrations, while acetone alone was added to control flasks. Incubations were continued for another 5 min. Results shown are means ± S.E. for triplicate slices from four animals in each experimental group (total $n = 8$ for statistical analysis by unpaired t-test). Results were similar when 0.1 mM methylnitro-nitrosoguanidine was added in 50 mM Tris (pH 7.4) rather than acetone.

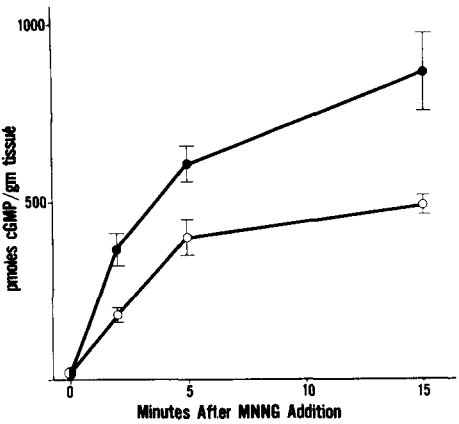
Experimental group	pmol cyclic GMP/g wet wt.		
	Control	0.1 mM methylnitro-nitrosoguanidine	3 mM methylnitro-nitrosoguanidine
Sham-operated	19 ± 2	350 ± 30	1680 ± 200
Hepatectomized	22 ± 3	630 ± 60 *	1810 ± 190
	pmol cyclic GMP/mg protein		
Sham-operated	0.13 ± 0.01	2.4 ± 0.2	11.4 ± 1.6
Hepatectomized	0.17 ± 0.02	4.7 ± 0.5 *	13.7 ± 1.4

* $P < 0.01$ compared to value for sham-operated group.

sponding values in liver slices from unoperated controls (not shown). When examined for a 15 min period after the addition of 100 μM methylnitro-nitrosoguanidine to slice incubates, cyclic GMP accumulation was enhanced at all time points in slices from regenerating liver (Fig. 1). By 30 min, cyclic GMP accumulation was falling toward basal in both groups, but remained higher in regenerating liver (not shown). Incorporation of [^{3}H]thymidine into DNA was stimulated 3.7 ± 0.3-fold by partial hepatectomy 24 h after surgery, and thus indicative of a significant regenerative response to the partial hepatic resection.

Fig. 2 compares cyclic GMP generation in whole hepatic homogenates of the two experimental groups prepared 24 h after operation. In the presence of 2.5 μM methylnitro-nitrosoguanidine the rate of generation of cyclic GMP was markedly enhanced in homogenates of regenerating liver as compared to sham operated controls. With this low concentration of methylnitro-nitrosoguanidine a lag phase of guanylate cyclase activation was evident in both groups, which is not seen with higher concentrations of methylnitro-nitrosoguanidine [40]. Table II compares basal and methylnitro-nitrosoguanidine responsive guanylate cyclase of whole homogenates, the 100 000 × g soluble and particulate hepatic fractions from normal and regenerating liver. Basal enzyme activities in whole homogenate and the soluble fraction of regenerating liver and the responses of these preparations to a maximally effective concentration of methylnitro-nitrosoguanidine (100 μM) did not differ significantly from the corresponding values for sham operated animals. By contrast, 2.5 μM methylnitro-nitrosoguanidine gave near maximal activation of whole homogenate and soluble guanylate cyclase from regenerating liver (35-fold increase), whereas this concentration produced only a 4-fold increase in controls (Table II).

Consistent with previous reports [23—25] basal particulate hepatic guanylate



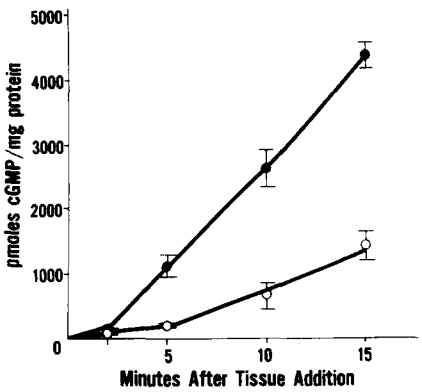


Fig. 1. Time course of cyclic GMP (cGMP) accumulation in liver slices incubated with submaximal (0.1 mM) methylnitro-nitrosoguanidine (MNNG). Assay conditions were identical to those for experiments described in Table I except that reactions were stopped at the indicated times after methylnitro-nitrosoguanidine addition. Zero-time slices were removed immediately before addition of methylnitro-nitrosoguanidine. Points represent mean ±S.E. of six slices from regenerating (•) and sham-operated livers (○) 24 h after operation.

Fig. 2. Time course of cyclic GMP (cGMP) generation in the presence of submaximal methylnitro-nitrosoguanidine during guanylate cyclase assay. Assay conditions were identical to those for experiments described in Table II except that reactions were stopped at the times indicated. Homogenates from regenerating (•) and sham-operated (○) livers were added at $t = 0$ to mixtures with a final methylnitro-nitrosoguanidine concentration of 2.5 μM. Each point represents the mean ±S.E. for determinations on four livers from each experimental group.

TABLE II

BASAL AND METHYLNITRO-NITROSOGUANIDINE RESPONSE GUANYLATE CYCLASE ACTIVITY OF LIVER HOMOGENATE AND SUBCELLULAR FRACTIONS FOLLOWING PARTIAL HEPATECTOMY OR SHAM OPERATION

Guanylate cyclase activities of whole homogenates, 100 000 × *g* soluble and particulate fractions were determined from the conversion of [α-^{32}P]GTP to cyclic [^{32}P]GMP. Incubations were conducted for 7 min in reaction mixtures containing 2.8 ± 0.2 and 2.6 ± 0.2 mg/ml homogenate protein, 1.03 ± 0.03 and 0.90 ± 0.03 mg/ml soluble protein, and 2.6 ± 0.2 and 2.3 ± 0.2 mg/ml particulate protein from livers of sham-operated and hepatectomized animal, respectively. Each value represents the mean ± S.E. of tissue preparations from the number of individual animals shown in parentheses. MNNG, methylnitro-nitrosoguanidine.

Fraction	Experimental group	pmol cyclic GMP/min per mg protein		
	[MNNG]:	0	2.5 μM	100 μM
Homogenate (4)	Sham-operated	15 ± 0	38 ± 1 **	690 ± 20 **
	Hepatectomized	17 ± 2	230 ± 60 *,**	630 ± 40 **
Soluble (8)	Sham-operated	54 ± 3	190 ± 20 **	2300 ± 100 **
	Hepatectomized	46 ± 2	1800 ± 100 *,**	2000 ± 100 **
	[MNNG]:	0	40 μM	200 μM
Particulate (5)	Sham-operated	2.5 ± 0.1	4.0 ± 0.6 **	9.1 ± 0.5 **
	Hepatectomized	7.0 ± 0.2 *	9.2 ± 1.1 *	22.3 ± 0.5 *,**

* $P < 0.01$ compared to value for sham-operated group.
** $P < 0.01$ compared to corresponding value without methylnitro-nitrosoguanidine.

cyclase activity was significantly higher 24 h after partial hepatectomy as compared to sham operation (Table II) and the particulate enzyme system of regenerating liver was less responsive to methylnitro-nitrosoguanidine and other *N*-nitroso compounds than was soluble activity [12]. As also shown in Table II, the absolute activity of the particulate fraction with maximal methylnitro-nitrosoguanidine was clearly greater in regenerating liver than in control liver. However, the relative responses to maximal methylnitro-nitrosoguanidine over the corresponding basal values were comparable in both groups. A concentration of 40 μM methylnitro-nitrosoguanidine, which produced submaximal activation of particulate enzyme from control livers, was ineffective in particulate fractions of regenerating liver. Response of particulate activity to 200 μM and 3 mM methylnitro-nitrosoguanidine were similar to each other and maximal. The basal and methylnitro-nitrosoguanidine responsive enzyme activities of the sham operated group shown in Table II did not differ detectably from corresponding values in unoperated controls.

Basal and methylnitro-nitrosoguanidine responsive guanylate cyclase were examined as a function of time during a 48 h period after operation (not shown). Basal particulate and methylnitro-nitrosoguanidine responsive soluble guanylate cyclase increased in parallel fashion following partial hepatectomy, being unchanged at 6 h and maximally elevated 24 h after operation. Since 98% of methylnitro-nitrosoguanidine responsive guanylate cyclase of hepatic homogenate was found in the soluble fraction and sensitivity of this fraction to submaximal methylnitro-nitrosoguanidine appeared to be selectively enhanced, this property of the soluble enzyme system of regenerating liver was further examined.

Effect of dilution and mixing of supernatant fractions on guanylate cyclase response to submaximal methylnitro-nitrosoguanidine

Dilution of the regenerating liver supernatant fractions, 4-fold, had no significant effect on the response of this preparation to 2.5 μM methylnitro-nitrosoguanidine (Table III). By contrast, dilution of control supernatant resulted in a clear increase in its response to 2.5 μM methylnitro-nitrosoguanidine. Furthermore, when supernatants from the two experimental groups were combined, the resultant guanylate cyclase response to 2.5 μM methylnitro-nitrosoguanidine was significantly lower than the mean of the individual responses, and did not differ significantly from that of control supernatant alone (Table III). Subsequent 4-fold dilution of the mixture of the two supernatants resulted in a response to methylnitro-nitrosoguanidine nearly identical to the predicted value (Table III). Results were similar using supernatants from sham operated or unoperated controls. Dilution or mixing of supernatant fractions did not alter responses to 100 μM methylnitro-nitrosoguanidine (not shown). These findings suggested the presence of factor(s) in control hepatic supernatants which suppress guanylate cyclase activation by a low concentration of methylnitro-nitrosoguanidine.

Investigation of the molecular weight range of soluble factors suppressing guanylate cyclase activation by submaximal methylnitro-nitrosoguanidine

The molecular weight of putative inhibitory factor(s) present in supernatant

TABLE III

EFFECTS OF DILUTION WITH BUFFER AND COMBINATION OF SUPERNATANTS FROM THE TWO EXPERIMENTAL GROUPS ON SUBMAXIMAL METHYLNITRO-NITROSOGUANIDINE RESPONSIVE GUANYLATE CYCLASE ACTIVITIES

Assay conditions were identical to those for the experiment described in Table II. Methylnitro-nitrosoguanidine was present at final concentration of 2.5 μM. Expected methylnitro-nitrosoguanidine responsive activities for combined supernatants were the average of the activity in the two component fractions. Values represent mean ± S.E. of determinations on eight livers from each experimental group. Hpx, hepatectomized.

Source of supernatant	pmol cyclic GMP/min per mg protein	
Protein (mg/ml):	1.1	0.28
Sham-operated	130 ± 30	470 ± 120 ***
Hepatectomized (Hpx)	1000 ± 200 *	1100 ± 100 *
Sham : Hpx-1 : 1		
Observed	220 ± 70	720 ± 110 ***
Expected	580 ± 110 **	760 ± 80

* $P < 0.001$ compared to sham-operated control.
** $P < 0.005$ compared to observed.
*** $P < 0.002$ compared to value at higher protein concentration.

of liver was examined by fractionation of supernatants on Sephadex G-25 columns and incubation of the high and low molecular weight column fractions with supernatant from regenerating liver. Since the high molecular weight fraction from the column contained guanylate cyclase activity, results are expressed as the ratio of activity with 2.5 μM methylnitro-nitroguanidine to that with maximal methylnitro-nitrosoguanidine (100 μM). Low molecular weight fractions from liver supernatants contained no assayable guanylate cyclase activity. As shown in Table IV, only high molecular weight fractions had inhibitory effects on guanylate cyclase activation by 2.5 μM methylnitro-nitrosoguanidine. Low molecular weight supernatant fractions from both experimental groups (Table IV) and from concentrated buffer (not shown) did not significantly affect the response to 2.5 μM methylnitro-nitrosoguanidine. The high molecular weight fraction from regenerating liver supernatant significantly inhibited the response to 2.5 μM methylnitro-nitrosoguanidine when added at the higher but not at the lower concentration tested. By contrast, the high molecular weight fraction from control liver supernatant significantly suppressed activation by 2.5 μM methylnitro-nitrosoguanidine at both concentrations. These results are consistent with the presence of soluble factor(s) with a molecular weight in excess of 5000 which suppress guanylate cyclase activation by submaximal concentrations of methylnitro-nitrosoguanidine. They further suggest that the factor(s) are present at higher concentration in control compared to regenerating liver.

Correlation of inhibitory activity of supernatant fractions with supernatant heme concentration

The average heme content of the high molecular weight fraction from supernatant of regenerating liver was 4 ± 1 μM versus 12 ± 1 μM in controls. In view of (a) recent reports of the inhibitory effects of hemeproteins on guanylate

TABLE IV

METHYLNITRO-NITROSOGUANIDINE ACTIVATION OF SOLUBLE GUANYLATE CYCLASE IN THE PRESENCE OF HIGH AND LOW MOLECULAR WEIGHT SEPHADEX G-25 FRACTIONS

Hepatic supernatant fractions and the sucrose-Tris homogenization buffer were eluted from 1.5 × 5 cm columns of Sephadex G-25 with 10 mM Tris-HCl, pH 7.4, at 4°C. The first 3.7 ml was designated the high molecular weight fraction (fraction 1). Two subsequent peaks with absorbance at 280 nm were noted. Fraction 2, eluted with 3.7—8.3 ml of Tris, contained among other components, sucrose as evidenced by osmolality changes when sucrose-Tris was passed through the column. Fraction 3, a second low molecular weight band, was eluted with 8.3—15.6 ml of Tris. The three fractions were lyophilized and dissolved in water to 1/4 the original volume applied to the column. Guanylate cyclase assays were initiated by addition of 25 μl of a mixture which contained 19 μl of supernatant fraction of perfused regenerating liver (see Table V), plus 3 or 6 μl of a G-25 fraction. An additional 3 μl sucrose-Tris buffer was added, where required. Results are mean ± S.E., with the 100 000 × *g* supernatant fractions from two different regenerating livers. MNNG, methylnitro-nitrosoguanidine.

Source of fraction	Fraction No. MNNG:	Volume (μl)	pmol cyclic GMP/min per mg protein		
				100 μM	2.5/100 μM
Supernatant from sham-operated rats	1	3.0	23 ± 2	86 ± 8	0.27 ± 0.05
		6.0	22 ± 0	117 ± 12	0.19 ± 0.02
	2	6.0	38 ± 7	41 ± 4	0.93 ± 0.13
	3	6.0	31 ± 7	36 ± 2	0.86 ± 0.09
Supernatant from hepatectomized rats	1	3.0	78 ± 2	89 ± 8	0.88 ± 0.04
		6.0	54 ± 13	136 ± 15	0.39 ± 0.10
Buffer	1	3.0	48 ± 3	49 ± 4	0.99 ± 0.07
		6.0	51 ± 2	55 ± 4	0.93 ± 0.05

cyclase activation [28,29], (b) the higher heme content of the high molecular weight fractions of control supernatant and (c) the obvious pallor of regenerating livers in vivo and in vitro, the possibility that enhanced guanylate cyclase responsiveness to methylnitro-nitrosoguanidine in this tissue might be related to altered heme content was assessed. The heme content of hepatic supernatant fractions from regenerating liver was lower (1.1 ± 0.1 μM, mean ±S.E.) than that of sham-operated controls (2.7 ± 0.4 μM, $P < 0.01$, $df = 15$). Fig. 3 depicts the relationships between heme concentrations of the enzyme assay mixtures and guanylate cyclase responsiveness to 2.5 and 100 μM methylnitro-nitrosoguanidine. As shown in Fig. 3A, the final heme concentration of assay mixtures resulting from addition of control hepatic supernatant fractions was 0.5 μM or greater in all but one instance (0.5—1.8 μM), and was associated with only a modest guanylate cyclase response to 2.5 μM methylnitro-nitrosoguanidine. By contrast, final heme content of assay mixtures containing supernatant fractions from regenerating liver was generally less than 0.5 μM (0.1 μM—0.55 μM). This correlated with enhanced responsiveness of guanylate cyclase to 2.5 μM methylnitro-nitrosoguanidine. As also shown in Fig. 3A, the addition of exogenous heme in the form of bovine hemoglobin to supernatant fractions from three regenerating livers, so that final concentrations in the enzyme assay mixture exceeded 0.5 μM, suppressed guanylate cyclase responses of these preparations to 2.5 μM methylnitro-nitrosoguanidine, compared to values observed in the same supernatants in the absence of exogenous heme. These results suggested that the higher endogenous heme content of control supernatant frac-

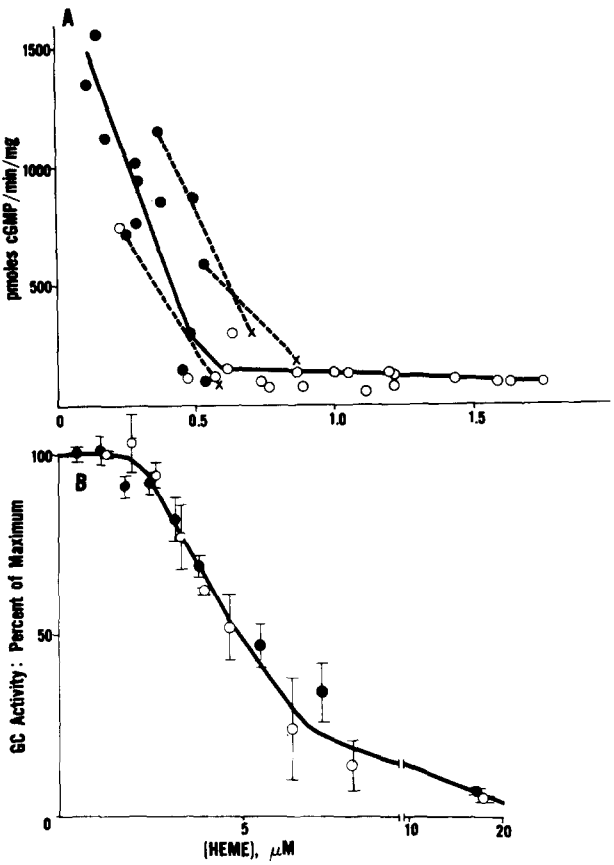


Fig. 3. Guanylate cyclase (GC) responses to 2.5 μM (A) or 100 μM (B) methylnitro-nitrosoguanidine as a function of the heme concentration of the enzyme assay mixture. (A) Heme was present in the final assay mixture either as a consequence of the endogenous heme content of sham-operated liver (○) or regenerating (●) supernatant fractions or as a result of addition of exogenous heme as bovine hemoglobin to three regenerating liver supernatants (X). In the latter instances, dashed lines connect results from the same supernatant assayed in the presence and absence of 0.33 μM exogenous heme in the final reaction mixture. Each point represents a determination from an individual liver. (B) Guanylate cyclase response to 100 μM methylnitro-nitrosoguanidine was examined in hepatic supernatant fractions from two sham-operated and two hepatectomized rats in the absence or presence of exogenous heme added as bovine hemoglobin. Final heme content of the assay mixtures were increased to the values shown by addition of 0.33—16.7 μM exogenous heme. The lowest heme concentrations shown represent endogenous heme. Each point is mean ±S.E. of determinations from two separate supernatant fractions. cGMP, cyclic GMP.

tions may play a role in suppression of the response to 2.5 μM methylnitro-nitrosoguanidine. As shown in Fig. 3B, responses of soluble guanylate cyclase of both control and regenerating liver to maximal methylnitro-nitrosoguanidine (100 μM) was also suppressed by addition of exogenous hemoglobin, but much higher final heme concentrations were necessary to demonstrate this effect. The soluble guanylate cyclase responses of both experimental groups to 100 μM methylnitro-nitrosoguanidine was inhibited 50% by approximately 5 μM heme in the final reaction mixture, and nearly abolished by heme concentration of 8 μM or greater.

Table V shows the effects of in situ perfusion of control and regenerating

TABLE V

EFFECT OF LIVER PERFUSION ON GUANYLATE CYCLASE RESPONSE TO METHYLNITRO-NITROSOGUANIDINE

Livers were perfused through the portal vein with 0.9% NaCl at 4°C until the effluent from the sectioned inferior vena cava was clear. Tissue was then processed as described in Materials and Methods and soluble guanylate cyclase activity assayed under conditions described in Table II. Heme concentrations, with and without parentheses, represent those in hepatic supernatant and in the final assay mixture, respectively.

	[Heme] (M)	pmol cyclic GMP/min per mg protein		
	methylnitro-nitrosoguanidine:	0	1 μM	2.5 μM
Perfused				
Sham-operated	0.31 ± 0.01 (0.39 ± 0.03)	48 ± 9	370 ± 40	1650 ± 90
Hepatectomized	0.14 ± 0.04 (0.43 ± 0.13)	42 ± 5	1100 ± 270	1880 ± 60
Non-perfused				
Sham-operated	0.63 ± 0.04 (1.9 ± 0.1)	39 ± 3	55 ± 3	104 ± 7
Hepatectomized	0.45 ± 0.03 (1.3 ± 0.1)	36 ± 3	81 ± 4	540 ± 30

liver on the endogenous heme content of the supernatant fraction and their responses to low concentrations of methylnitro-nitrosoguanidine. In situ perfusion of livers lowered the endogenous heme content of both groups compared to non-perfused values, presumably due to removal of red cells. At these lower endogenous heme concentrations, soluble guanylate cyclase response to 2.5 μM methylnitro-nitrosoguanylate was comparable for the two experimental groups. This is in contrast to the findings in non-perfused livers. As also shown in Table V, the response to 1 μM methylnitro-nitrosoguanidine was similar in supernatant fractions of non-perfused control and regenerating livers, but exhibited much greater relative enhancement in fractions from the latter group following perfusion. This effect correlated with the lower residual heme content of regenerating liver after perfusion.

Discussion

The results indicate that stimulation of the guanylate cyclase-cyclic AMP system of regenerating rat liver by low concentrations of methylnitro-nitrosoguanidine is enhanced compared to that of liver from sham-operated or unoperated controls. The increase in cyclic GMP accumulation in slices of regenerating liver exposed to submaximal methylnitro-nitrosoguanidine was observed in the presence of MIX, a potent inhibitor of cyclic nucleotide phosphodiesterase activity [41]. This finding, as well as the enhanced sensitivity of guanylate cyclase to activation by submaximal methylnitro-nitrosoguanidine in homogenates of regenerating liver strongly imply, but do not conclusively prove, that increased cyclic GMP accumulation in the slice reflects an accelerated rate of nucleotide generation. The increased sensitivity of whole homogenate guanylate cyclase of regenerating liver to submaximal methylnitro-

nitrosoguanidine was attributable to changes in the responsiveness of the soluble enzyme system.

The enhanced response of soluble guanylate cyclase of regenerating liver to methylnitro-nitrosoguanylate was probably due to reduced levels of heme proteins which suppressed the action of methylnitro-nitrosoguanidine. This conclusion was supported by: (a) selective enhancement of the guanylate cyclase responses of control supernatant fractions to methylnitro-nitrosoguanidine upon tissue dilution (Table III); (b) reduced response of combined supernatant fractions from control and regenerating liver to methylnitro-nitrosoguanidine compared to the value expected from individual responses (Table III); (c) the greater inhibitory activity of a high molecular weight fraction of supernatant of control versus regenerating liver on the response of soluble guanylate cyclase to methylnitro-nitrosoguanidine (Table IV); (d) correlation of enhanced sensitivity of soluble guanylate cyclase to methylnitro-nitrosoguanidine with a lower endogenous heme content of the supernatant fraction of regenerating liver (Fig. 3A) and its suppression by addition of an amount of exogenous hemoglobin which raised the heme content to levels observed in control supernatants (Fig. 3B) and (e) increased methylnitro-nitrosoguanidine responsiveness of supernatant guanylate cyclase from control livers following the lowering of heme content by in situ perfusion (Table V).

An inhibitory effect of hemoglobin on activation of soluble guanylate cyclase by methylnitro-nitrosoguanidine, azide, nitroprusside and nitric oxide has been reported by Miki and coworkers [28] and by Mittal et al. [29]. In contrast, work from this laboratory [30,31,36] has demonstrated biphasic effects of heme on the activation of partially purified guanylate cyclase by methylnitro-nitrosoguanidine, nitric oxide, nitroprusside or nitrite. Addition of low concentrations of heme or hemeprotein to partially purified enzyme is required for expression of the actions of methylnitro-nitrosoguanidine and related agonists, and is correlated with formation of the paramagnetic nitrosyl heme complex [30,31,36]. By contrast, high concentrations of heme or hemeprotein or, alternatively, a high ratio of heme to agonist concentration results in suboptimal enzyme activation [31,36]. The molecular basis for these biphasic effects of heme is at present uncertain. However, heme binds to partially purified guanylate cyclase [31], and thus restoration of enzyme responsiveness by addition of a low concentration of heme may reflect repletion of a critical prosthetic group lost during enzyme purification. Conversely, at high ratios of heme to agonist, competition of unliganded heme or hemeproteins for nitric oxide with heme sites on the enzyme essential for guanylate cyclase activation may be operative [36]. The results of the present study show that the endogenous hemeprotein concentration of supernatant fractions from nonperfused control livers (3—5 μM) is sufficient to suppress the guanylate cyclase response at methylnitro-nitrosoguanidine concentrations which are maximally effective at the diminished hemeprotein content of regenerating liver. By analogy, the increased cyclic GMP accumulation in response to methylnitro-nitrosoguanidine in slices of regenerating liver may reflect a decreased heme content of this tissue.

Enhanced responsiveness of the guanylate cyclase system of regenerating liver to methylnitro-nitrosoguanidine occurs between 6 and 24 h after partial

hepatectomy. Since this correlates temporally with the time of increased susceptibility of liver to the carcinogenic action of *N*-nitroso compounds [5], a relationship between these two phenomena is possible. However, guanylate cyclase is known to be activated by noncarcinogenic *N*-nitroso compounds such as *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide [32], as well as by other noncarcinogenic nitric oxide donors [30,31]. Accordingly, activation of this enzyme per se cannot be central to the carcinogenic actions of *N*-nitroso compounds. Nevertheless, such considerations do not exclude the possibility that the cyclic GMP response to methylnitro-nitrosoguanidine, which is enhanced in regenerating liver, represents an event that participates in expression of its oncogenic effect.

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